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Selection of salt tolerant embryogenic line in *Jatropha curcas* L., which has potentiality of biodiesel

Chọn lọc dòng mô phôi soma chịu mặn của cây cọc rào (Jatropha curcas L.*), một loài cây có tiềm năng về nhiên liệu sinh học*

Research article

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The embryogenic calli were grown on MS medium containing NaCl with concentrations from 50 to 300 mM. After 2 weeks of culture, salinity tolerance threshold was identified at 150 mM NaCl. Higher concentrations of NaCl stimulated a significant reduction in the calli survival rate and the highest rate was 78.67% at 50 mM. After subculturing callus to the embryo culture medium containing NaCl, the growth and embryogenesis were not affected at the concentrations of 50 – 100 mM. Especially, at 50 mM NaCl the embryogenesis rate reached 83.33%. In contrast, 150 mM NaCl inhibited the somatic embryogenesis. After 4 weeks, culturing somatic embryos on medium MS with addition of 0.07 mg/l spermidin at 50 – 100 mM NaCl, the embryogenesis was considered good and embryos developed through several stages: globular, heart, torpedo and cotyledonary. However, at 150 mM NaCl the globular stage appeared in the culture process. The process of morphohistology and using dye carmine – iod and acridine orange observed the structure of generative callus and embryos at several stages.

Mô sẹo có khả năng phát sinh phôi được nuôi cấy trong môi trường có chứa muối NaCl với nồng độ thay đổi từ 50 – 300 mM. Sau 2 tuần nuôi cấy, chúng tôi xác định được ngưỡng chịu mặn của mô sẹo có khả năng sinh phôi cây Cọc rào là 150 mM. Nồng độ muối NaCl càng cao thì tỷ lệ sống của mô sẹo giảm dần và đạt giá trị cao nhất là 78,67% tại nồng độ 50 mM NaCl. Khi chuyển mô sẹo sang môi trường phát sinh phôi có chứa muối NaCl với nồng độ thay đổi, chúng tôi thấy ở nồng độ muối NaCl 50 – 100 mM không ảnh hưởng đến khả năng sinh trưởng và phát sinh phôi, đặc biệt là tại nồng độ 50 mM NaCl giúp kích thích sự hình thành phôi từ mô sẹo với tỷ lệ hình thành phôi đạt 83,33%. Ngược lại, nồng độ từ 150 mM NaCl gây ức chế quá trình hình thành phôi soma từ mô sẹo. Tiếp tục khảo sát ảnh hưởng của muối đến khả năng phát triển và nảy mầm của phôi soma. Ghi nhận kết quả sau 4 tuần nuôi cấy phôi soma trong môi trường MS có bổ sung 0.07 mg/l spermidin, tại nồng độ 50 – 100 mM NaCl khả năng hình thành phôi tốt và phôi phát triển qua các giai đoạn phôi hình cầu, hình tim, hình thủy lôi và hình lá mầm. Đặc biệt ở nồng độ 50 mM số lượng phôi lá mầm đạt giá trị cao với 13,33 phôi. Nồng độ muối NaCl 150 mM chỉ xuất hiện phôi hình cầu trong suốt thời gian nuôi cấy. Quá trình giải phẫu hình thái phôi và sử dụng thuốc nhuộm 2 màu carmin – iod và acridine orange đã cho thấy rõ hơn về cấu trúc mô sẹo có khả năng sinh phôi và phôi hình thái.

Keywords: embryogenic callus, somatic embryo, salt tolerant, *Jatropha curcas* L., morphohistology

1. Introduction

In recently years, high temperature, flood, drought, thunderstorm, river erosion, rising sea level, salinization appears non cyclically and effect strongly on our lives, especially the historic salinization, that prove the climate change is increasingly complicated without rule. In there, salinization is one of the interesting issues. The salinization phenomenon is encroaching inland, that impacts strongly

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on soil, plant, as well as the economic life of the people. Therefore, besides soil improvement, the improvement of plant varieties resistant to salt tolerance is an imperative solution. Salinity is a factor that effects on crop yields, especially irrigation. There were about 400 million hectares of saline ground (Flowers *et al.*, 1977). The increase of salinity in soil has been one of serious issues that threats the survivability of crops.

Micropropagation technique is a multipurpose tool to study the behavior of cells as well as the whole plant under stress conditions. The exploitation of somaclonal variation is also useful for *in vitro* selection of cells and tissues against several stresses (Bajaj, 1987; Tal, 1996). In spite of limitations, salt-tolerant cell lines and plants were reported in some species, such as tomato (Hassan and Wilkins, 1988), wheat (Barakat and Abdel-Latif, 1996), rice (Lutts *et al.*, 1999), sunflower (Alvarez *et al.*, 2003).

Jatropha can be grown in the arid soil, harsh climate, furthermore, this plant is an economic fuel and environmental friendliness, so creating a salinity tolerance Jatropha line is one of the solutions to solve the urgent problems mentioned above. Vietnam is one of the countries that interested in the research and development of Jatropha to produce biodiesel. Jatropha brings a promise of biofuels that does not compete with other crops. In addition, Jatropha is used as medicines and has many advantages in environmental and economic benefits.

In this report, we focus to sutdy the effect of NaCl on the development of embryogenic callus and shoot of *J. curcas*. Then, selection of salt tolerant embryogenic line in *Jatropha curcas* L.

2. Materials and methods

Embryogenic callus of *Jatropha curcas* L., which was created and multiplicated at National Key Laboratory of Plant cell technology, Institute of Tropical Biology, was used as materials.

In this study, basal MS medium was used. Depending on experiments, plant growth regulators and NaCl with different concentrations were supplemented.

2.1 Effects of the concentrations of NaCl on the development of embryogenic callus of *J. curcas*

Embryogenic callus was cut into pieces and cultured on basal MS supplemented with 1.0 mg/l kinetin, 1.5 mg/l 2,4-D (Do Dang Giap *et al.*, 2012) and NaCl with different concentrations (0, 50, 100, 150, 200, 250, 300 mM).

Observation target: Survival rate of callus (%) after 2 weeks of culture.

2.2 Effects of NaCl on somatic embryogenesis of *in vitro J. curcas*

Embryogenic callus was cut into pieces and cultured on basal MS (Murashige and Skoog, 1962) supplemented 1.0 mg/l kinetin (Do Dang Giap *et al.*, 2012) and NaCl with different concentrations within salinity tolerance threshold (50, 100, 150 mM).

Observation target: Somatic embryogenesis rate (%); Number of embryos after 4 weeks of culture.

2.3 Effects of NaCl on the development and shoot formation of *J. curcas* somatic embryos

Somatic embryos were cultured on basal MS medium supplemented with 0.07 mg/l spermidine (Do Dang Giap *et al.*, 2013) and NaCl (50, 100, 150 mM). The materials were subcultured on suitable embryogenesis medium.

Observation target: Total number of embryos; Number of globular embryos, heart embryos, torpedo embryos, cotyledonary embryos.

Statistical analysis

The experiments were arranged in single factor, complete randomized design. The data are recorded and analyzed by MSTATC, using LSD multiple range test.

3. Results

3.1 Effects of the concentrations of NaCl on the development of embryogenic callus of *J. curcas*

Table 1 showed the difference between treatments with and without (control treatment) NaCl. The survivability of embryogenic callus of *J. curcas* decreased when increasing the concentrations of NaCl, or survival rate of embryos was inversely propotional with the concentrations of NaCl. In control treatment, embryogenic calli grew and developed with 100% of alive embryos. At 50, 100 and 150 mM NaCl supplemented treatments, survival rate of embryos decreased to 78.67%, 57.00%, 49.00%, respectively. The higher concentrations of NaCl (200, 250 and 300 mM), the lower the survival rate of embryos (23.67%, 12.33%, 4.00%, respectively).

Table 1. Effects of NaCl at different concentrations on the survivability of embryogenic callus of *J. curcas*

Concentrations of NaCl (mM)	Survival rate of embryos (%)
0	100.00[a]
50	78.67[b]
100	57.00[c]
150	49.00[d]
200	23.67[e]
250	12.33[f]
300	4.00[g]

The different letters (a, b, c, ...) in the same column show significant differences at α = 0.01 in the LSD multiple range test.

Observing treatments supplemented NaCl with higher concentrations (over 150 mM), the survivability of embryogenic callus strongly decreased, this proved that 150 mM of NaCl concentration was the salinity tolerance threshold of embryogenic callus of *J. curcas* (Fig 1).

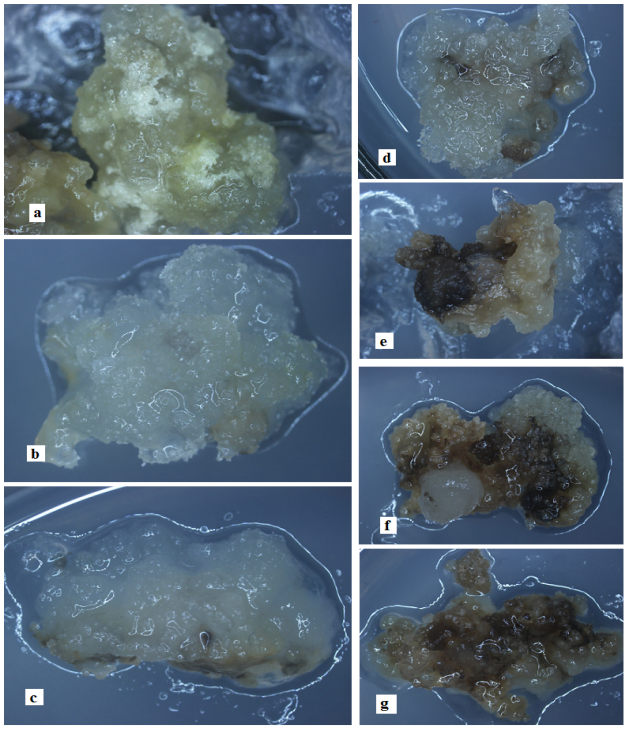

Figure 1. Embryogenic calli form on the selective medium at different concentrations of NaCl.
a. 0 mM; b. 50 mM; c. 100 mM; d. 150 mM; e. 200 mM; f. 250 mM; g. 300 mM

The results of callus anatomy showed that embryogenic cells had big nucleus and concentrated cytoplasm. Nucleus and contents of cytoplasm have the affinity for carmine and stain dark pink; non embryogenic cells with big vacuole and small nuclear stain dark blue when staining by carmine-iodine. Selecting calli growing on selective media of NaCl at different concentrations from 50 to 150 mM (Fig 2) to anatomise, red fragmented cells with integrity nucleus were collected (Fig 2d). These cell structures were similar to those on control treatment and cells color showed they were alive and growing.

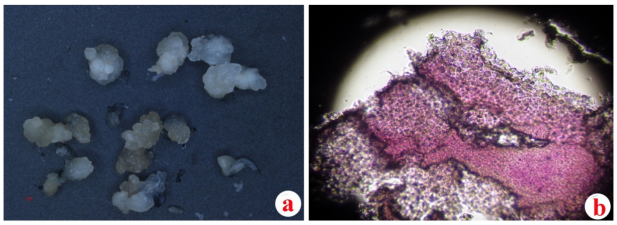

Figure 2. Morphological and anatomical structure of embryogenic callus developed on selective medium at concentrations of 50 – 150 mM NaCl.

After 2 weeks of culture on saline selective medium, calli had some induction with salinity stress. To determine cell survivability, acridine orange was used. Under Fluorescence microscopy, by glowing ability of cell nucleus, red-orange color defined dead cells and green defined alive cells. The anatomy results (Fig 3) showed that at the high concentrations of NaCl, dead cell rate was also high; at 150 mM of NaCl (Fig 3d) there were rare dead cells, and the increase of concentrations of NaCl from 200 mM to 250 mM (Fig 3e, f) obtained ratio of dead ascended and at treatment supplemented 300 mM of NaCl (Fig 3g) most of calli died.

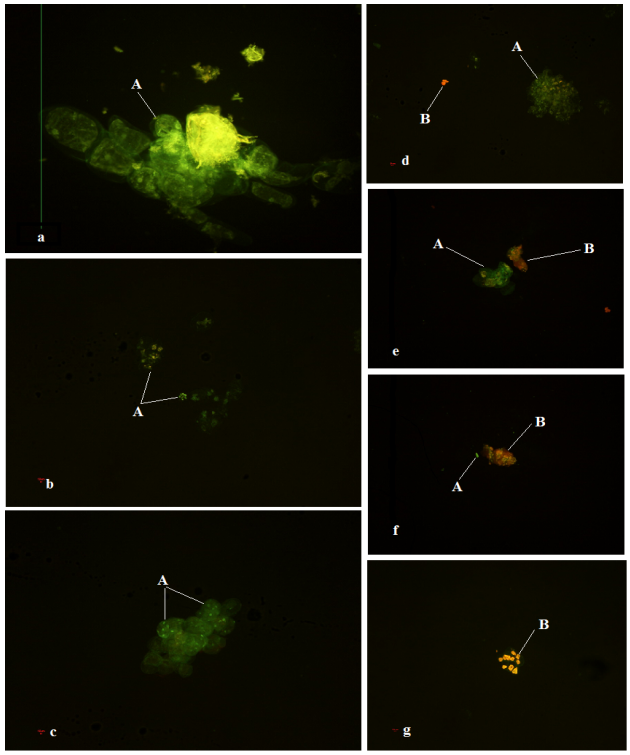

Figure 3. Cells were stained with acridine orange.
a. Cells in control treatment; b, c, d, e, f, g. Callus cells in additional treatments of NaCl at concentrations 50, 100, 150, 200, 250 and 300 mM, respectively. A. Alive cell (green); B. Dead cell (red-orange).

3.2 Effects of NaCl on somatic embryogenesis of *in vitro J. curcas*

The results showed that treatments with different concentrations of NaCl led to different somatic embryogenesis ability, rate of embryo induction and number of embryos. The highest result was recorded on treatment supplemented with 50 mM NaCl. When subcultured embryogenic callus on embryo inducting medium, somatic embryos had some quickly differentiated expression on culture medium. Somatic embryos develop on four main stages: globular shape, heart shape, torpedo shape and cotyledonary shape. Treatment supplemented with 50 mM NaCl obtained the highest ratio of total number embryos (80.00%). Higher concentrations of NaCl inhibited somatic embryogenesis process, that caused the ratio of inducting embryo lower than 50.00% (26.67%) at treatment supplemented with 150 mM NaCl. Treatment of 50 mM NaCl stimulant somatic embryogenesis derived from embryogenic callus, opposite to that, when supplementing 150 mM NaCl into culture medium, it inhibited that process (Fig 4).

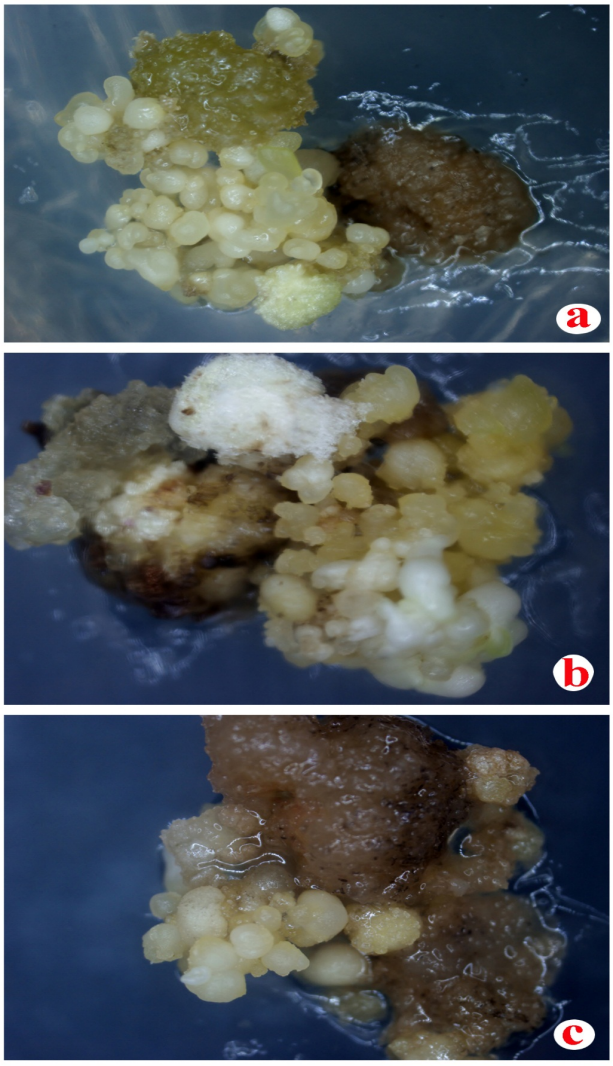

Figure 4. Effects of NaCl on somatic embryogenesis of callus. *a. 50 mM; b. 100 mM; c. 150 mM*

Table 2. Effects of NaCl on somatic embryogenesis derived from callus of *in vitro J. curcas*

Concentrations of NaCl (mM)	Somatic embryogenesis rate (%)
50	**80.00^{a}**
100	40.00^{b}
150	26.67^{b}

The different letters (a, b, c, ...) in the same column show significant differences at α = 0.01 in the LSD multiple range test.

3.3 Effects of NaCl on the development and shoot formation of J. curcas somatic embryos

At treatment that the concentration of NaCl was 150 mM, most of embryos exited as globular shape. Though, at treatment that the concentrations of NaCl were 50 mM, 100 mM and control treatment, embryos developed by globular stage, heart stage, torpedo stage and cotyledonary shape. Especially at 50 mM NaCl, the average number of heart embryos and cotyledonary embryos were the highest ones (48 and 13.33, respectively) (Table 3, Fig 5). That confirmed when supplementing 150 mM NaCl, it would exhibit the growth and germination, embryos kept their globular shape through culture progress. At the concentrations of 50 mM and 100 Mm NaCl, there are no effects on the growth of embryos. The same result was in the report of Marroquín *et al.* (2011) when surveying the effects of NaCl on embryo formation of Habanero pepper salt tolerance at 75 – 300 mM of NaCl in culture medium.

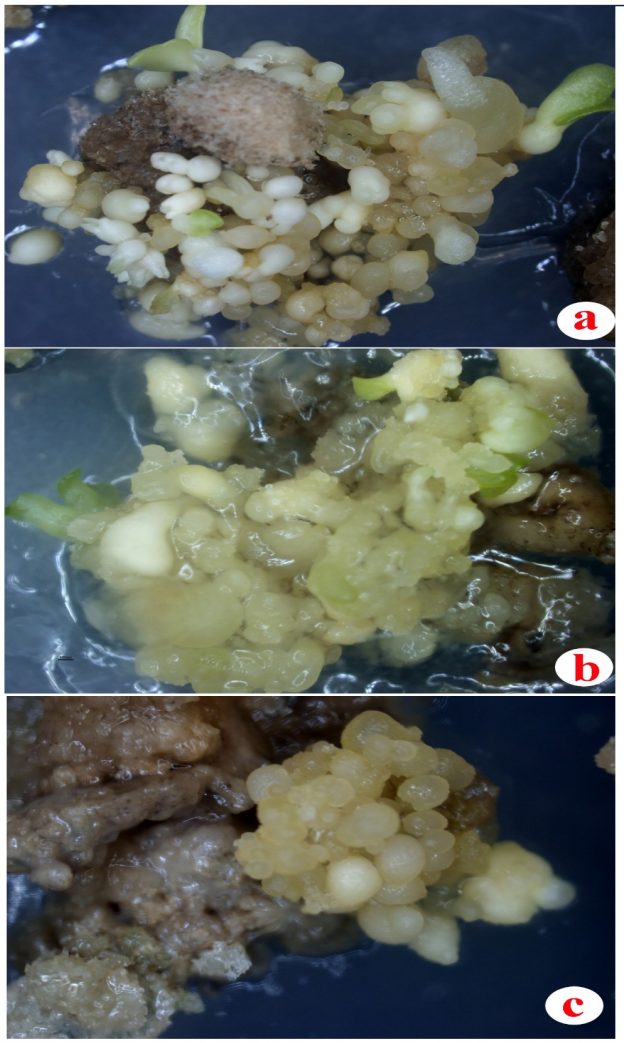

Figure 5. Effects of NaCl on the development of somatic embryos. *a. 50 mM; b. 100 mM; c. 150 m*

Table 3. Effects of NaCl on the development of somatic embryos of *J. curcas*

Concentrations of NaCl (mM)	Total number of embryos	Number of globular embryos	Number of heart embryos	Number of torpedo embryos	Number of cotyledonary embryos
0	87.67^{a}	15.33^{b}	30.67^{b}	32.00^{a}	9.67^{ab}
50	**90.67^{a}**	**11.00^{b}**	**48.00^{a}**	**18.33^{b}**	**13.33^{a}**
100	64.00^{b}	9.67^{b}	32.67^{b}	15.33^{b}	6.33^{b}
150	42.67^{c}	42.67^{a}	0.00^{c}	0.00^{c}	0.00^{c}

The different letters (a, b, c, ...) in the same column show significant differences at α = 0.01 in the LSD multiple range test.

4. Discussion

As the results at 3.1, there was difference between treatments with NaCl and control treatment. The survivability of embryogenic callus decreased while increasing NaCl concentration, or survival rate of embryogenic callus inversely proportional to NaCl concentration. In control treatment, embryogenic callus grew well and gained the highest survival rate (100%), treatments supplemented with NaCl at different concentrations had lower survival rate (table 1). In this study, the salinity threshold of *J. curcas* was 50 – 150 mM. However, the concentrations of salinity impacts on different plants were different. Croughan *et al.*, (1978) determined the salinity threshold of *Medicago sativa* cell was 1% (w/v) NaCl. While Greetha and Rao (1997) studied on *Vigna radiata* L., they selected salt tolerance callus line and salinity threshold was determined at 300 mM NaCl. On potato plant, Ochatt *et al.* (1999) showed that calli which were cultured on medium containing 60 mM to 450 mM NaCl grew well. Cotton plants has salinity threshold at 10 g/l NaCl, but their embryos could bear the salinity at 15 g/l NaCl. This could be explained that each plant has different genotypics, different structure of cell wall and metabolism ability at each stage, which leads to the difference of absorption capacity, metabolism and abiotic stress tolerance level as well as salt tolerance pressure. Saline pressure of NaCl caused some changes in metabolism process of embryogenic callus, especially effecting on the osmotic ability. The osmotic ability of embryogenic callus decreased significantly when increasing the concentrations of NaCl in culture medium. The osmotic ability is one of the important parameter which is effected by abiotic stress, such as drought, salinity (Marroquín *et al.*, 2011).

After determining salinity threshold of of embryogenic callus was 50 – 150 mM, this threshold was used to survey the embryogenesis. The result of Table 2 showed that at higher concentration of NaCl (150 mM), it inhibited somatic embryogenesis process. Treatment supplemented with 50 mM NaCl obtained the highest ratio of total number embryos (80.00%). In contrast, higher concentrations of NaCl inhibited somatic embryogenesis process, that caused the ratio of inducting embryo lower than 50.00% (26.67%) at treatment supplemented with 150 mM NaCl (Fig 4). The same thing was proved in some studies of Bekheet *et al.* (2006) on *Allium cepa* L., Habanero pepper (Marroquín *et al.*, 2011). Saline pressure of NaCl strongly effects on ion exchange capacity, especially K^+ and Na^+. The increase of NaCl concentration, K^+ content of cells decreased, while Na^+ concentration ascended significantly. The accumulation of Na^+ in issues under saline pressure seems to be main factor later than the adverse impact of salt on nutrient absorption and development (Kumar *et al.*, 2008).
In the process of embryo development of *J. curcas*, at treatment that the concentration of NaCl was 150 mM, most of embryos exited as globular shape. Though, at treatment that the concentrations of NaCl were 50 mM, 100 mM and control treatment, embryos developed by globular stage, heart stage, torpedo stage and cotyledonary shape. Especially at 50 mM NaCl, the average number of heart embryos and cotyledonary embryos were the highest ones (48 and 13.33, respectively) (Table 3, Fig 5). That confirmed when supplementing 150 mM NaCl, it would exhibit the growth and germination, embryos kept their globular shape through culture progress. At the concentrations of 50 mM and 100 Mm NaCl, there are no effects on the growth of embryos. The same result was in the report of Marroquín *et al.* (2011) when surveying the effects of NaCl on embryo formation of Habanero pepper salt tolerance at 75 – 300 mM of NaCl in culture medium. This could be explained by saline pressure factor, because of the directive effects on osmotic ability of cells, which leads to changing the metabolism. High concentrations of NaCl inhibit embryos growth and bipolar structure capacity of embryos, but lower concentrations of NaCl or non NaCl in medium, embryos grow normally (Mukherjee *et al.*, 2003). Bipolar structure capacity of embryos is requisite of embryo germination process. Biosynthesis pathway that produces galactinol, fructan, trehalose, ononitol, proline and glycine betaine to regulate osmotic pressure worked on enhancing abiotic stress tolerance capacity (Qin et al., 2011).

5. Conclusion

Salinity threshold of of embryogenic callus was 50 – 150 mM. Treatment supplemented with 50 mM NaCl obtained the highest ratio of total number embryos (80.00%). At 50 mM NaCl, the average number of heart embryos and cotyledonary embryos were the highest (48 and 13.33, respectively).

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